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Review article

A review on goat sperm cryopreservation☆

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Abstract

The ability to cryopreserve spermatozoa from all of the domestic species is challenging. Even though all of the cells must endure similar physical stresses associated with the cryopreservation processes, sperm from the different species are very different in size, shape and lipid composition, all of which affect cryosurvival. Thus, when a cryopreservation protocol has been optimized for sperm of one species, it may not be ideal for sperm of other species. Bovine and caprine sperm-freezing diluents, for example, contain similar ingredients, but interactions between goat seminal plasma and egg yolk are deleterious to the sperm, a situation not observed with bovine seminal plasma and egg yolk. Therefore, a thorough understanding of the specifics of sperm freezing from a particular species will improve the cryosurvival of sperm from that species. This review updates information relating to the cryopreservation of goat semen, with emphasis on the peculiarities specific to the species. The topics discussed include the effects of goat seminal plasma during cryopreservation, sperm dilution and concentration, freezing and thawing methodologies, the components of cryopreservation diluents, and traditional and recently investigated cryoprotectants. In addition, suggestions for creating a standardized freezing protocol for goat semen are also presented.
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1. Introduction

The cryopreservation of mammalian sperm is a complex process that involves balancing many factors in order to obtain satisfactory results. To insure even minimal success, not only proper diluent, sperm dilution rate, cooling rate and thawing rate are required, but also an intricate knowledge of the sperm physiology for the species is essential to maximize post-thaw recovery of sperm and consequently the fertility. Goat sperm is an excellent example of this because even though there are many similarities between goat sperm and sperm from other domestic species, such that similar types of cryopreservation media, cryoprotectants, and freezing and thawing rates can be used to cryopreserve these sperm, goat sperm require unique attention to maximize the post-thawing viability. For example, the deleterious interaction between egg yolk and the bulbourethral gland secretions exists for goat semen that does not exist for other species, such as the bull, boar, or ram

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(Roy, 1957; Iritani and Nishikawa, 1963; Iritani et al., 1964).

Due to these and other differences between the sperm of the domestic species, this review discusses seminal plasma effects, semen cryopreservation media, cryoprotectants, semen dilution, cooling rate, and thawing rate specific to the caprine species. Previous review articles cover different aspects of this topic (Amoah and Gelaye, 1997; Chemineau et al., 1999; Leboeuf et al., 2000).

2. General semen handling

2.1. Seminal plasma

The most common cryopreservation diluents used for goat semen contain either egg yolk or non-fat dried skim milk. However, the dilution of goat semen into diluents containing egg yolk or milk can be detrimental to the sperm cells. The harmful interactions between seminal plasma and egg yolk were first documented by Roy (1957) and with milk, by Nunes et al. (1982). Roy (1957) observed that sperm cells maintained their motility in egg yolk diluents if the seminal plasma was removed, but if neat semen was added to egg yolk media, the egg yolk coagulated and the sperm died. It was also determined that egg yolk coagulated due to an enzyme of bulbourethral origin, thus named the egg yolk-coagulating enzyme (EYCE). Similarly, Nunes et al. (1982) identified a protein (SBUIII) from the goat bulbourethral gland, which decreased survival of cooled or frozen goat sperm diluted in milk-based media. It also induced the acrosome reaction and subsequent cell death of spermatozoa incubated in milk medium at 37 °C (Pellicer-Rubio et al., 1997).

It is likely that EYCE and SBUIII is the same molecule (Leboeuf et al., 2000). The EYCE was identified as phospholipase A (Iritani and Nishikawa, 1961, 1963), and SBUIII as a 55–60 kDa glycoprotein lipase from the goat bulbourethral gland (BUSgp60) (Pellicer-Rubio et al., 1997). EYCE acts as a catalyst that hydrolyzes egg yolk lecithin into fatty acids and lysolecithin (Iritani and Nishikawa, 1961, 1963). This hydrolysis causes the sperm membranes to be more fusogenic thereby inducing the acrosome reaction (Upreti et al., 1999), and chromatin decondensation (Sawyer and Brown, 1995), which is toxic to the sperm (Aamdal et al., 1965). BUSgp60 lipase has structural homology to porcine pancreatic lipases (Carrière et al., 1994) and, similar to EYCE, BUSgp60 is responsible for hydrolysis of plasma membrane triglycerides and triglycerides in the skim milk that result in fatty acid production (lysolecithin production with egg yolk, and oleic acid with milk triglycerides) which is toxic to sperm (Pellicer-Rubio et al., 1997; Pellicer-Rubio and Combarnous, 1998). Regardless of the mechanisms of EYCE and BUSgp60, their activation in diluents is detrimental to the quality of the sperm cells during cooling and cryopreservation (Pellicer-Rubio et al., 1997; Pellicer-Rubio and Combarnous, 1998).

The conventional method of overcoming the harmful interactions of seminal plasma and egg yolk or milk proteins is to dilute the goat semen sample in a buffered diluent and then separate the seminal plasma from the sperm by centrifugation. Cells are washed either once or twice, each for 10–15 min at 550–950 × *g* (Nunes et al., 1982; Ritar and Salamon, 1982; Memon et al., 1985; Singh et al., 1995; Leboeuf et al., 1998). Washing semen samples, however, is a time-consuming process that may damage cells if performed improperly but, if performed correctly, can be beneficial. The benefits of seminal plasma removal thus vary. Certain research indicates that removing seminal plasma is necessary for maximizing post-thaw motility and acrosomal integrity in goat semen (Drobnis et al., 1980; Ritar and Salamon, 1982; Memon et al., 1985), while others report positive results for sperm frozen without washing (Ritar and Salamon, 1982; Azerêdo et al., 2001). Alternative diluents that minimize the sperm and lipase interactions have been proposed, including adding BUSgp60 lipase inhibitors, using lipid-free cow milk, a triglyceride-free diluent containing the milk protein casein, or using milk from species other than dairy cows where fatty acid and triacylglycerol structure differ so that the enzymatic reactions do not occur (Pellicer-Rubio and Combarnous, 1998). Kundu et al. (2000, 2001, 2002) demonstrated that goat sperm can be cryopreserved in egg yolk and milk-free media, but this work was performed on cauda epididymal sperm, not ejaculated cells.

3. Sperm dilution/concentration

It is essential that a semen sample be diluted properly so that there are sufficient numbers of sperm and

sufficient diluent to accommodate the cells in an insemination straw, so that a high fertility rate can be achieved using the least number of inseminations and the lowest number of sperm per insemination.

Historically, semen samples in farm animals have been diluted by either diluting semen with specific volumes of diluents or by diluting semen to a specific spermatozoa concentration. Dilution rates of 1:1–1:23 (v/v; semen to diluent) have been used successfully (Evans and Maxwell, 1987; Ritar et al., 1990a,b). Perhaps a better way of diluting semen, for comparison purposes, is based on the sperm concentration. Reports of sperm being successfully frozen, and reasonable fertility being achieved, have been obtained with samples ranging from 80 to 500×10^6 cells/ml (Corteel, 1974; Ritar et al., 1990a,b; Karatzas et al., 1997).

4. Freezing and thawing semen samples

Diluted buck semen is cooled to 4–5 °C over 1.5–4 h and then frozen in either pellets or straws (Evans and Maxwell, 1987; Chemineau et al., 1991; Tuli et al., 1991; Ritar, 1993; Gravance et al., 1997; Leboeuf et al., 2000).

Freezing of sperm in pellets is rapid and inexpensive, but inventory management is problematic because the actual semen samples can not be labelled. Once the semen sample is cooled, semen aliquots of 0.1–0.3 ml are dispensed into indentations on a block of dry ice (solid carbon dioxide; −79 °C) and frozen for 2–4 min. The pellets are then plunged into liquid nitrogen for storage (Evans and Maxwell, 1987; Chemineau et al., 1991).

The freezing of sperm in straws is more expensive and laborious than the pellet technique, but each sample can be labeled for accurate inventory management. After diluting and cooling the semen samples, the sperm are loaded into 0.25 or 0.5 ml straws, placed on a rack, and frozen in liquid nitrogen vapor; the temperature of which varies by the height above the liquid nitrogen. A styrofoam box containing liquid nitrogen or a programmable freezer that controls the cooling rate have both been successfully used to cryopreserve the goat sperm. When using a styrofoam box, the rack containing the samples is placed into the liquid nitrogen vapor at a height of 3–4 cm above the liquid for 7–8 min and the straws are then plunged in liquid nitrogen for storage (Evans and Maxwell, 1987). Alternatively, Chemineau et al. (1991) reported straw size should determine the freezing height above the liquid nitrogen. It was suggested that 0.5 ml straws should be frozen 4 cm above liquid nitrogen for 5 min, and then plunged into the liquid nitrogen, while 0.25 ml straws should be placed 16 cm above the liquid nitrogen for 2 min, lowered to 4 cm for 3 min, and plunged into the liquid nitrogen for storage. However, other freeze heights and times have been quoted, e.g., 4–5 cm above liquid nitrogen for 4–5 min, with acceptable results (Gravance et al., 1997; Leboeuf et al., 2000).

Programmable freezers are convenient for the freezing of large quantities of semen straws and for controlling the rate of freezing. These freezers may be used to replicate pellet freezing by placing the semen straws in a freezer at −80 °C for 7–15 min and then plunging the semen straws into liquid nitrogen (Evans and Maxwell, 1987; Blash et al., 2000). The benefit of many programmable freezers is that the freezing curve can be customized, e.g., 4 to −5 °C at 4°/min, −5 to −110 °C at 25°/min and −110 to −140 °C at 35°/min, and then the semen straws can be plunged into liquid nitrogen (personal communication, IMV Corporation, USA, 2004).

Ritar et al. (1990a,b) reported sperm frozen in pellets to yield superior motility following thawing (39%) compared to sperm frozen in straws. However, cell motility was similar for sperm frozen in 0.25 (33% post-thaw motility) and 0.5 ml straws (34% post-thaw motility). Differences in post-thaw motility, viability and fertility may be attributed to the different cooling rates produced by the pellet and straw methods (Ritar, 1993). However, the choice of cryopreservation method used should also take into account other factors, such as ease of handling, labor, insemination technique, and inventory management.

Thawing sperm samples is determined by the method used to freeze the sperm. Sperm pellets should be thawed in a dry test tube at 37 °C while the thawing of straws may be performed using various methods (Evans and Maxwell, 1987). Traditionally, a straw is thawed by placing it in a 37 °C water bath for 12–30 s (Deka and Rao, 1987). This method is superior (36.1% mean motility across four diluents after 4 h of incubation) to a slow thaw method (18.9% motility), where the semen straw is placed in a 5 °C water bath for 2 min (Deka and Rao, 1987). Increasing the thawing

temperature to 70 °C and thawing the semen straw for only 7 s resulted in significantly higher progressive motility (36.9%) and plasma membrane integrity (39.8%) compared to thawing straws at 37 °C for 2 min (31.5%, 33.7%; progressive motility and plasma membrane integrity, respectively), or 40 °C for 20 s (32.4%, 33.5%; progressive motility and plasma membrane integrity, respectively) ($P < 0.05$; Tuli et al., 1991). Attention to temperature and timing becomes much more critical at temperatures greater than 37 °C, as these high temperatures can result in tremendous sperm mortalities if performed improperly (Tuli et al., 1991).

5. Cryopreservation diluents and cryoprotectants

5.1. Diluents

The purpose of a cryopreservation diluent is to supply the sperm cells with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for the spermatozoa to survive temporarily. Logically, each of the different components comprising the media have been investigated separately, and in combination, to maximize the post-thaw sperm viability and fertility. In general, a goat sperm cryopreservation medium includes a non-penetrating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant (glycerol, ethylene glycol, or dimethyl sulfoxide), a buffer (Tris or Test), one or more sugars (glucose, lactose, raffinose, saccharose, or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Evans and Maxwell, 1987).

A non-fat dried skim milk diluent (Corteel, 1974) or a Tris–glucose diluent (Salamon and Ritar, 1982) are most commonly used for cryopreserving goat sperm. Modifications of these diluents have been investigated with varying results (Evans and Maxwell, 1987; Singh et al., 1995; Keskintepe et al., 1998; Blash et al., 2000). These studies have tried to answer the ever-present questions of whether there are optimum cryopreservation media, does buck sperm have a preference for any one particular medium, and what the optimal concentration of the constituent is. To address these issues, specifics about goat semen cryopreservation media (osmolality, pH and buffers, sugars, cryoprotectants) are presented so that their effects on goat sperm are understood and consequently their inclusion or exclusion from a diluent.

5.2. Osmolality

Goat spermatozoa survive cryopreservation and remain fertile in media composed of a wide range of constituents. Many different sugars, salts and buffers, for example, can be included in varying molar concentrations in the cryopreservation medium, without injuring the spermatozoa. While the osmolality of the medium may vary within limits, goat spermatozoa prefer a hyperosmotic medium for cryopreservation. Salamon and Ritar (1982) observed that the optimal tonicity of a Tris–glucose–citric acid goat sperm medium to be 948 kPa (540 mOsm). The conclusion that this is the optimal tonicity for this medium was determined by comparing combinations of Tris buffer and sugars, in varying osmotic concentrations, which ranged between 737 and 1112 kPa (Salamon and Ritar, 1982). Furthermore, Bowen et al. (1988) reported that less damage occurred to goat sperm when frozen in diluents having osmolalities ranging between 425 and 525 mOsm, compared to media having an osmolality of either 325 or 625 mOsm.

5.3. pH and buffering systems

Large changes in semen pH can result in sperm damage, infertility, or sperm mortality. Therefore, in order to sustain the viability and fertilizing ability of sperm it is essential to maintain a proper environment by controlling the pH fluctuations in the cryopreservation media. One of the functions seminal plasma plays is to buffer the sperm cells against changes in pH, but this is meant to occur in vivo and not under in vitro conditions which occur during cryopreservation (Mann, 1954). For this reason, substances that buffer pH changes are routinely included in cryopreservation media to minimize changes. In general, a buffering solution for goat sperm media should have a pK_a of 7.0 (pH range of 6.0–8.0), be water soluble and membrane impermeable, have minimal interactions with salts, be minimally affected by buffer concentration, temperature and ionic contents on the dissociation of the buffer, have metal complexing properties, and be able to withstand enzymatic and non-enzymatic degradation (Good et al., 1966; Graham et al., 1972).

Although many investigators have determined the effects of pH on buck sperm, the pH of a cryopreservation medium is not frequently reported. However, the pH of egg yolk or milk-based goat sperm diluents, should normally range between 6.75 and 6.8 (Ritar and Salamon, 1982; Salamon and Ritar, 1982; Deka and Rao, 1987; Tuli and Holtz, 1992; Chauhan et al., 1994). Fukuhara and Nishikawa (1973) observed diluent pH to be very important for maximizing goat sperm respiration and motility. Oxygen uptake of goat sperm is maximal between pH 7.2 and 7.5 and sperm cell motility is optimal between a pH of 7.0 and 7.2, indicating that an optimal medium for goat sperm survival in vitro should be 7.2 (Fukuhara and Nishikawa, 1973). This is logical because mammalian semen normally has a pH in the range of 7.2–7.8 (Prins, 1999). Jaiswal and Majumder (1998) observed that altering the extracellular pH increases the intracellular pH and initiates forward motility in the goat caput-epididymal sperm. Increasing the medium pH from 7 to 8 stimulates about 50% of cauda epididymal sperm to become motile, indicating a sensitivity of the sperm to pH fluctuation (Jaiswal and Majumder, 1998). In addition, increasing intracellular pH activates downstream or parallel pathways that activate protein kinase A, an indicator of capacitation in mammalian sperm cells (Visconti and Kopf, 1998).

The concentration of the buffering additives also affects goat sperm viability. Salamon and Ritar (1982) investigated tris(hydroxyl-methyl)aminomethane (Tris) buffer across a range of concentrations and recorded an interaction between Tris concentration and the sugars present in the medium. Post-thaw motility was greater with the Tris buffer, at any concentration (300, 375, or 450 mM), being combined with 21, 42, or 62 mM glucose (mean total motility of all glucose concentrations, 33%) or fructose (33%). The addition of lactose (29%) or raffinose (17%) resulted in lower percentages of motile cells (Salamon and Ritar, 1982). It was concluded that under the conditions tested, buck sperm prefer fructose or glucose, when combined with Tris, and that the concentration of the sugars required is very small (0–62.4 mM) for successful cryopreservation (Salamon and Ritar, 1982). Other findings reported greater cryoprotective effects for monosaccharides than disaccharides, when used in combination with Tris (Molinia et al., 1994a). Furthermore, goat sperm can survive a broad range of Tris concentrations (300–600 mM), but the total osmolality of the solution is important. The combination of sugar and Tris buffer (737–1112 kPa) impacted on the success of the cryopreservation medium (Salamon and Ritar, 1982).

While the previous research investigated optimizing the Tris buffer concentration, Tuli and Holtz (1992) tried to determine which zwitterion buffer, Bes, Tes, Test, or Tris, was most compatible with buck semen in a cryopreservation medium. The experiments used an egg yolk–fructose diluent that varied, in the type of buffer, but were all titrated to a pH of 6.75. Significantly greater progressive motility (44%) and percentage live cells (49%) were observed in semen samples frozen in the Tris–citric acid buffer, compared with Bes, Tes, and Test buffers (25, 20, and 23%; 31, 27, and 29%, progressive motility and live cells, respectively) ($P < 0.01$), but no differences were recorded in glutamic oxaloacetic transaminase (GOT) release for any of the diluents analyzed (Tuli and Holtz, 1992). Other research also reports the benefits of Tris buffer in the cryopreservation media for post-thaw motility, when compared with Tes-yolk, skim milk, egg yolk citrate, and raffinose–egg yolk diluents (Drobnis et al., 1980; Deka and Rao, 1987).

The question still remains regarding the mechanism by which these buffers affect the sperm. Many theories relating to the action of the buffers have been proposed, but it is generally believed that buffers aid in the cellular dehydration process by creating an osmotic force (Molinia et al., 1994b), thereby increasing the physical stability of the sperm cell plasma membrane, and neutralizing acids generated during in vitro storage (Ijaz et al., 1989). Some results on buffer research are surprising, particularly regarding the wide use and positive results found with Tris. As stated earlier, Tris seems to be the buffer of choice for use with buck sperm, but this is not the case in other species where negative aspects of the buffer, such as increased capacitation and acrosome reaction rates, and swelling of the apical ridge of the sperm cell, have been documented (Molinia et al., 1996; Ijaz et al., 1989). In other mammalian species (bovine and ovine) Tris is a poor buffer particularly below a pH of 7.5, where it is unable to withstand temperature fluctuations. The pH of the media can decrease as much as 1 pH unit when Tris media is warmed from 0 to 37 °C (Graham et al., 1972; Ijaz et al., 1989; Molinia et al., 1994b). Still, positive results have been reported with Tris in goats, bulls and rams, but because the pK_a values of this and all buffers are

different, direct comparisons are not completely valid (Good et al., 1966). This is not an all inclusive list of buffering compounds that have been tested for cryopreservation of goat sperm, but it illustrates the rationale for inclusion of one of these compounds in a medium and the reason for the popularity of the Tris buffer.

5.4. Sugars

It is logical to include sugars in a cryopreservation diluent, as seminal plasma contains sugars. Goat sperm readily utilizes fructose, glucose, lactose and other sugars for respiration, and these sugars also provide osmotic balance and cryoprotection, but of all the sugars, fructose has the greatest molar concentration in neat goat semen (Mann, 1954; Corteel, 1973; Salamon and Ritar, 1982; Aboagla and Terada, 2003). The semen diluents contain a variety of sugars, with a wide range of osmolalities (6–375 mM) (Corteel, 1974; Ritar and Salamon, 1982; Salamon and Ritar, 1982; Evans and Maxwell, 1987). The choice of which sugar to include in a diluent may be based on the functionality of the chemical. Fructose is the primary substrate for glycolysis in goat seminal plasma, so it is logical to include this sugar in the medium (Pellicer-Rubio et al., 1997). Likewise, glucose is an excellent substrate in goat sperm metabolism and is essential for providing energy so that the sperm cells can function in a normal physiological manner (Fukuhara and Nishikawa, 1973; Corteel, 1974).

Corteel (1974) demonstrated that goat sperm froze better in a milk diluent if the seminal plasma was removed from the sperm. It was thus necessary that this cryopreservation medium contain glucose so that a readily available source of energy for the spermatozoa is available and results in a greater sperm motility (30% motility with glucose, compared to 10% motility without glucose; $P < 0.05$) following cryopreservation (Corteel, 1974). These results are in agreement with Fukuhara and Nishikawa (1973) who described the role of seminal plasma, namely, its purpose to provide energy and protection to the sperm cells. Therefore, these same criteria are necessary for a cryopreservation medium, particularly when using sperm cells that have been washed of their seminal plasma.

Up to this point, discussion of sugars has been limited to low-molecular weight molecules (fructose and glucose) that can pass through the plasma membrane of a sperm. Various effects can be observed when sugars that are not capable of diffusing across a plasma membrane, such as lactose, sucrose, raffinose, trehalose, or dextrans are added to the diluent. In these instances, the sugars create an osmotic pressure, inducing cell dehydration and therefore, a lower incidence of intracellular ice formation. These sugars also interact with the phospholipids in the plasma membrane, reorganizing the membrane which results in sperm that is better suited to surviving the cryopreservation process (Molinia et al., 1994a; Aisen et al., 2002). Unlike the simple sugars glucose and fructose, these disaccharides act primarily as cryoprotectants. More recently, trehalose has been included in goat sperm cryopreservation diluents. Aboagla and Terada (2003) reported that trehalose acted as a cryoprotectant in the Tris–citric acid–glucose (TCG) diluent developed originally by Salamon and Ritar (1982). Washed goat sperm were diluted and frozen in the TCG medium containing 0, 93.75, 187.5, 281.25 or 375 mM trehalose (Aboagla and Terada, 2003). The post-thaw motility of sperm frozen in the presence of 375 mM trehalose resulted in significantly greater total sperm motility (78%), progressive motility (61%) and path velocity (96 μ/s), compared to the 0 mM trehalose treatment (62%, 49%, 81 μ/s, respectively) ($P < 0.05$). Also, flow cytometric evaluation of the membrane fluidity of the goat sperm before cryopreservation revealed the plasma membranes of goat sperm treated with trehalose to be more fluid at 38 °C, compared to the plasma membranes of untreated goat sperm (Aboagla and Terada, 2003). The exact mechanism by which trehalose and other non-penetrating sugars affect the sperm membrane is unknown, but it is theorized that these sugars penetrate into the plasma membrane of the spermatozoa and form hydrogen bonds with the polar headgroups of the phospholipids (Liu et al., 1998; Aboagla and Terada, 2003). The insertion of these disaccharides into the membrane limits the amount of dehydration that can occur, and consequently the physical damage due to cell volume changes associated with freezing and thawing (Liu et al., 1998). Trehalose also causes an increase in membrane fluidity due to protein and phospholipid reorganization, a suppression of the injurious effects of the membrane lipid phase transition, and accordingly, a dehydration of cells at temperatures, lower than if a disaccharide had not been used (Aboagla and

Terada, 2003). Together, this means cell damage is minimized due to less intracellular ice formation and consequently more viable cells being recovered following cryopreservation (Aboagla and Terada, 2003).

6. Cryoprotectants

A cryoprotectant is included in a cryopreservation medium to minimize the physical and chemical stresses resulting from the cooling, freezing, and thawing of sperm cells. Cryoprotectants are classified as either penetrating or non-penetrating. A penetrating cryoprotectant is membrane permeable and acts intra- and extracellularly. Penetrating cryoprotectants are solutes, which cause dehydration of spermatozoa due to the osmotically driven flow of water, which varies according to compound. After short periods of time the cryoprotectant and water equilibrate and result in similar intracellular and extracellular concentrations (Amann, 1999). As the sperm cell now has less intracellular water, the freezing point of the cell is decreased and less intracellular ice formation will occur which is beneficial, because intracellular ice results in cell death, and consequently reduced fertility of the semen sample. The penetrating cryoprotectants also cause membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures, and therefore an increased ability to survive cryopreservation (Holt, 2000). Additionally, penetrating cryoprotectants are solvents that dissolve sugars and salts in the cryopreservation medium. A non-penetrating cryoprotectant cannot cross the sperm plasma membrane and therefore, only acts extracellularly (Amann, 1999). Therefore, a non-penetrating cryoprotectant may modify the plasma membrane of a cell, or act as a solute and lower the freezing temperature of the medium (Amann, 1999).

Many membrane-permeable cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol), and their combinations, have been tested with buck sperm (Ritar et al., 1990a,b; Tuli and Holtz, 1994; Singh et al., 1995; Kundu et al., 2000; Leboeuf et al., 2000), but the most frequently used penetrating cryoprotectant is glycerol. The addition of glycerol may be performed in a 1-, 2- or 3-step methodology at either 37 °C or 5 °C (Corteel, 1974; Salamon and Ritar, 1982; Tuli and Holtz, 1994; Leboeuf et al., 2000). The final concentration (v/v) of a penetrating cryoprotectant in a medium varies, but is determined by the toxicity of the chemical, and its beneficial effect on the spermatozoa. Glycerol, dimethyl sulfoxide (Me_2SO), and ethylene glycol are generally used in a range of 1–8%, but the greatest recovery of sperm post-thawing has been achieved with glycerol (Tuli and Holtz, 1994; Singh et al., 1995; Kundu et al., 2000). Combinations of cryoprotectants, such as glycerol and Me_2SO, have also been used, and yielded positive results. Kundu et al. (2000) observed that using glycerol (6%) alone as the cryoprotectant, resulted in higher percentages of motile sperm following cryopreservation (35%), compared to sperm frozen using ethylene glycol (13%) or Me_2SO (21%). The use of both glycerol (6%) and Me_2SO (5.9%) achieves a synergistic effect of the cryoprotectants. The post-thaw motility using glycerol and Me_2SO media separately was 33% and 15%, respectively, while the combination of the cryoprotectants resulted in 45% progressively motile spermatozoa (Kundu et al., 2001).

Addition of glycerol can induce osmotic damage to spermatozoa, but the extent of the damage varies according to the species. However, goat spermatozoa are reasonably tolerant to these osmotic conditions and can withstand a rapid exposure to glycerol. Trials testing step-wise addition of glycerol have yielded variable results, depending on the temperature at which the glycerol is added. Salamon and Ritar (1982) reported a single-step glycerol addition to semen samples at 30 °C to result in more motile spermatozoa, compared to a 2-step glycerolization at 30 °C, or a 2-step glycerolization at decreasing temperatures (glycerol dilution at 30 °C and dilution at 5 °C). Tuli and Holtz (1994) reported a step-wise method of glycerolization at 37 °C to result in similar proportions of progressively motile and live sperm, compared to a 1-step glycerolization at 37 °C, or a step-wise dilution at 5 °C—but the step-wise method of glycerolization at 37 °C had significantly less GOT release (99 units/ml) after thawing compared to the other methods (105 and 108 units/ml, respectively; $P < 0.05$). From these results, it appears that glycerol can be added to sperm in a single step.

The most common non-penetrating cryoprotectants used are egg yolk (2–20%) (Ritar and Salamon, 1982; Tuli and Holtz, 1994) and non-fat skim milk (10%, w/v) (Corteel, 1974; Leboeuf et al., 1998). Recent

research has also identified other membrane impermeable chemicals that may be used as cryoprotectants. Kundu et al., (2001), using cauda epididymal sperm and an egg yolk and glycerol-free medium, demonstrated that amino acids (L-proline, L-alanine, glycine or L-glutamine; 100–150 mM) could be used as cryoprotectants in goat sperm cryopreservation media and yield better post-thaw results (8–14% forward motility and 11–19% total motility recovery), compared to a control group (0% motile cells with 0 mM of amino acids). The amino acids exerted an even greater protection when combined with glycerol (0.87 M) and dimethyl sulfoxide (0.76 M) which resulted in post-thaw progressive motility of 50–55% (Kundu et al., 2001). Dextrans (10–2000 kDa) may also be used as non-penetrating cryoprotectants in the same egg yolk and glycerol-free medium, and yield 23 and 25% total and progressive motility, post-thawing (Kundu et al., 2002). The greatest percentages of forward (58%) and total sperm motility (60%) were recorded with 6.27 mM of the 10 kDa dextran in combination with glycerol (0.87 M) and dimethyl sulfoxide (0.76 M) compared to the control (22% forward motility and 25% total sperm motility). Kundu et al. (2002) hypothesized the combination of glycerol, Me_2SO and the dextran to result in an additive beneficial effect during cryopreservation, because of the molecular masses of the different chemicals. Glycerol and Me_2SO are membrane permeable and therefore, will dehydrate the spermatozoa and minimize the intracellular ice formation. As the dextran is membrane impermeable, its effect will be greatest outside the sperm cell, most probably by interrupting ice formation extracellularly (Kundu et al., 2002). Consequently, this combination of chemical substances results in cryoprotective action that is both intracellular and extracellular.

A complete understanding of the mechanism by which penetrating and non-penetrating cryoprotectants function is not completely understood, but the most recent research shows promise for expanding the understanding of how cryoprotectants function, and should therefore, improve current freezing methodologies.

7. Conclusions

An immediate need for the United States goat breeder community is a combination of practices similar to those used in France (Leboeuf et al., 1998) and those of the Certified Semen Services applied to bulls (NAAB, 2001), that describe culling of animals and semen processing. Culling of animals should be based on both breed standards (conformation), and semen quality (before and after cryopreservation), which will result in increases in product quality and reproductive performance. Caution should be exercised so that producers are not inadvertently decreasing genetic diversity within a breed by culling.

The body of research documents that there are many acceptable methods for cryopreserving goat semen. The focus of research should now be on maximizing fertility with a minimum number of sperm following cryopreservation. To accomplish this goal, alternative goat semen cryopreservation media, such as those investigated by Kundu et al. (2000, 2001, 2002), must be investigated further. Exploitation of methodologies such as these will require minimum processing where no centrifugation to remove seminal plasma will be necessary. This should result in greater numbers of viable cells following cryopreservation, due to less physical damage being inflicted on the sperm due to processing. If fewer sperm are used for insemination, then this will result in increased economic advantage to producers by more insemination doses being produced per ejaculate. In addition, less time spent processing semen will also result in greater efficiency for the producer and consequently greater profits.

Until the new methodologies have been thoroughly investigated, the use of the Tris–egg yolk cryopreservation diluents, such as the one described by Salamon and Ritar (1982), is recommended. The Tris–egg yolk diluents are beneficial because of their ease of use; in particular because centrifugation of semen is not necessary (Ritar and Salamon, 1982), a one-step dilution may be used (Salamon and Ritar, 1982), and preparation of the diluent is simpler because the heating step required with skim milk is not necessary. Other research by Tuli and Holtz (1992, 1994) testifies to the use of this type of goat semen cryopreservation diluent, but the composition of the diluent utilized in their research differs, in particular, by egg yolk concentration. Ritar and Salamon (1982) recommend using a diluent with 1.5% egg yolk, final concentration, while the diluent utilized by Tuli and Holtz (1992) contained 16.8% egg yolk, final concentration. The point to this comparison is that not all Tris–egg yolk diluents are the same and caution should be exercised concerning the ingredients

of the diluent, particularly because higher concentrations of egg yolk (>1.5%) decrease the post-thaw viability of buck semen samples that were not washed free of seminal plasma prior to cryopreservation (Ritar and Salamon, 1982).

The fertility of buck semen frozen in pellets and straws is similar (Ritar et al., 1990a,b); therefore it is recommended that buck semen be frozen in straws so that inventory management can be more precise. To maximize the post-thaw recovery of motile and viable buck sperm frozen with the Tris–egg yolk diluent, Ritar et al. (1990) determined that the semen straws should be placed 4 cm above liquid nitrogen for 4.5 min and then plunged into the liquid nitrogen for storage. This same freezing methodology can be adapted to programmable freezers by creating a cooling curve based on the temperature in a straw that is frozen in the afore-mentioned manner and the temperature observed every 30 s for the duration of the freeze. The buck semen straws should be thawed for 20–30 s in a 37 °C water bath.

The present state of goat-assisted reproductive technologies shows a great deal of promise. It is possible to successfully utilize frozen-thawed goat sperm for artificial insemination, IVF and embryo production, but the results presented in literature are quite variable. To overcome this variability the scientific community should endeavor to reach a consensus that addresses generally accepted practices for freezing goat semen (medium, cooling/freezing rate, sperm concentration, etc) and its subsequent uses across breeds. This will develop a deeper understanding of goat sperm physiology and enable more valid comparisons of research because the handling and freezing of the goat sperm will be identical.

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